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Invited Review

Toxicological importance of human biomonitoring of metallic and metalloid elements in different biological samples

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ABSTRACT

Human biomonitoring has become an important tool for the assessment of internal doses of metallic and metalloid elements. These elements are of great significance because of their toxic properties and wide distribution in environmental compartments. Although blood and urine are the most used and accepted matrices for human biomonitoring, other non-conventional samples (saliva, placenta, meconium, hair, nails, teeth, breast milk) may have practical advantages and would provide additional information on health risk. Nevertheless, the analysis of these compounds in biological matrices other than blood and urine has not yet been accepted as a useful tool for biomonitoring. The validation of analytical procedures is absolutely necessary for a proper implementation of non-conventional samples in biomonitoring programs. However, the lack of reliable and useful analytical methodologies to assess exposure to metallic elements, and the potential interference of external contamination and variation in biological features of non-conventional samples are important limitations for setting health-based reference values. The influence of potential confounding factors on metallic concentration should always be considered. More research is needed to ascertain whether or not non-conventional matrices offer definitive advantages over the traditional samples and to broaden the available database for establishing worldwide accepted reference values in non-exposed populations.

1. Introduction

Although the presence of a xenobiotic in the environment always represents a risk for living organisms, the onset of toxicity needs to consider key factors such as physicochemical properties of the compound, routes of exposure, health status, genetic susceptibility, etc. that are determinants of the reaction of the organism against harmful chemicals. Biomarkers provide useful information on the nature and the effect of an exposure, as well as on the susceptibility of individuals or populations to the toxic effects of such an exposure. However, this review will focus only on biomarkers of human exposure to metal and metalloid elements. Human biological monitoring has become an important tool in environmental and public health for the assessment of internal doses of harmful substances and to evaluate temporal changes in populations exposed to a particular environmental contaminant (Gil and Hernández, 2009; Hernández et al., 2014).

Toxic metals and metalloids are contaminants of great significance because they are widely distributed in air, water, soil and other environmental compartments as a result of anthropogenic or geological releases. The term "heavy metals" has been used inconsistently in the scientific literature and in legislation related to chemical hazards and the safe use of chemicals, thus creating confusion and misunderstanding. This term has never been defined by IUPAC, and there is a tendency to assume that the so-called "heavy metals" have highly toxic properties, so it should be abandoned and replaced by metal-ions (Nieboer and Richardson, 1980) or metallic elements. Moreover, this term has no coherent scientific basis as it refers to a metal and all its compounds, thus implying that they all have the same physicochemical, biological and toxicological properties, which is not certainly true (Duffus, 2002). On the other hand, although arsenic is not a metal, it has been often included under the term "heavy metal" which is totally inappropriate. Arsenic (As) is an element that has the physical appearance and properties of a metal, but it behaves chemically like a non-metal (Duffus, 2002). For the purpose of this review, we will use the term "metallic and metalloid elements" which is intended to cover major toxic metal and metalloid compounds.

The contamination chain of metallic and metalloid elements resulting from anthropogenic sources usually follows a cyclic order: industry, atmosphere, soil, water, foods and humans. According to biomonitoring data from the Centers for Disease Control (CDC) and other US biomonitoring studies, people are widely exposed to metallic and metalloid elements (COEH, 2011). Human exposure to these compounds may occur occupationally, environmentally, or through

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dietary intake, with food and water being the most common sources of exposure in the general population (ATSDR (Agency for Toxic Substances and Disease Registry), 2014). Hence, this contamination has raised great environmental concern because of its potential long-term effects on human health (Armah et al., 2014).

Some metallic and metalloid elements present well known toxic properties such as neurotoxic effects (including neurodevelopmental disorders, impaired cognition and intelligence, hyperactive behavior, decreased motor function), but they also can act as mutagenic and carcinogenic agents, endocrine disrupters, etc.

Exposure to low-dose of metallic and metalloid elements in non-occupational settings, together with their accumulative capacity in target organs, is becoming a serious problem, especially for pregnant women, breast-feeding mothers, elderly people and children as they are considered as the most vulnerable subgroups of population (Gil and Pla, 2001; Grandjean and Landrigan, 2014). Children are exposed to metallic and metalloid elements from early prenatal stages because of mother's exposure and the mobilization of these compounds from maternal tissues during pregnancy and breastfeeding. Exposure continues during childhood and pre-adolescence through food and water intake, inhalation of airborne pollution and/or dermal absorption of metallic and metalloid elements (Counter and Buchanan, 2004; Rodríguez-Barranco et al., 2013). In addition, the body burden of certain of these elements usually increases with advancing age as a result of their slow elimination from the body, as occurs with cadmium (Cd) and lead (Pb) (Gil, 2012; Grandjean et al., 1994). Nevertheless, elimination of metallic elements in humans varies considerably, from days to years, depending on their half-lives and the organ in which they accumulate, among other factors.

Early knowledge of the health effects of toxic metallic and metalloid elements is based on workers occupationally-exposed to relatively high levels in industry or in populations living in heavily polluted environments. Only in the last few years have studies concerning human biomonitoring (HBM) addressed the possible effects of chronic low environmental exposure to mixtures of these compounds in the general population of industrialized countries, especially those particularly susceptible, such as adolescents (Interdonato et al., 2014).

2. Biological samples useful for human biomonitoring

Biomonitoring has the advantage of providing unequivocal evidence that both exposure and uptake have taken place. Biological samples in HBM should be easily accessible under routine conditions and without health risk for the individual. For these reasons, blood and urine samples are the most widely used and accepted matrices for evaluating metallic and metalloid element levels in the human body in occupational and environmental toxicology.

Other less invasive biological samples, including saliva, placenta, meconium, hair, nails, teeth or breast milk have different toxicokinetic profiles and may prove to have practical advantages over classical biological fluids for the assessment of the internal dose of metallic and metalloid elements in exposed individuals (Table 1). Nevertheless, certain samples (e.g., hair) should be viewed only as a supportive tool and the analytical results put into perspective with other more reliable data (e.g., blood or urine concentrations) (Angerer et al., 2007; Harkins and Susten, 2003).

However, strict quality assurance during sampling and chemical analysis is extremely important. Analytical procedures must be standardized to help ensure more accurate and reliable results, so an adequate analytical validation of the methods is absolutely necessary.

The German Human Biomonitoring Commission has recommended two criteria to assess exposure: reference values and HBM values (Schulz et al., 2007). The reference values indicate the upper margin of background exposure to a given contaminant in a given population at a given time. By contrast, HBM values derive two different kinds of values: (a) HBM I, which represents the concentration of a substance in human biological material below which there is no risk for adverse health effects and, consequently, no need for action; (b) HBM II, which represents the concentration above which there is an increased risk for adverse health effects and then there is a need of reduced exposure. The latter can be considered as an intervention or action level. Adverse health effects should be considered for concentrations in the range between HBM I and HBM II (Schulz et al., 2007).

2.1. Blood

Metallic elements in blood are distributed between the non-cellular (plasma/serum) and intra-cellular compartments (essentially erythrocytes) and these compounds have different affinity for each compartment, depending on chemical properties (e.g. lead and erythrocytes). The serum/plasma fraction is the one filtered in the glomeruli, and therefore these compounds might accumulate in erythrocytes in subjects with poor kidney function. Thus, kidney function has a major physiological impact on the distribution of metallic elements between the red blood cell and serum compartment, resulting in concentrations higher in whole blood than in serum (Schultze et al., 2014).

Blood, as the traditional matrix for HBM of chemicals clearly reflects recent exposure to these compounds (Table 1). Whole blood must be taken with special tubes and bottles for metallic element measurements and vacutainer needles used for venipuncture should not add measurable levels of these elements to the collected blood. Furthermore, currently available anticoagulants have drawbacks as most of them are either polyanions (e.g., heparin) or metal chelators (e.g., EDTA or citrate) and therefore have a high affinity for metals (De Cremer, 2003). If an anticoagulant is used, it must be rigorously controlled, and heparin is the most frequently used for metallic and metalloid elements analysis. Recent exposures to Cd, Pb and mercury (Hg) by any route (digestive, respiratory and dermal) can be assessed by determining their levels in blood; however, a positive finding does not necessarily have to be related with any adverse effects (D'Ilio et al., 2013).

Analysis of Pb in whole blood is the most common and accurate method of assessing Pb exposure as blood Pb levels reflect recent exposure. The extensive use of blood Pb as a dose metric indicates the greater feasibility of incorporating blood Pb measurements into clinical or epidemiological studies, compared to other potential dose indicators, such as Pb in kidney, plasma, urine, or bone. Hg levels in the blood provide more useful information after recent exposures than after long-term exposures (ATSDR -Agency for Toxic Substances and Disease Registry-, 2007). Except for methylmercury, blood is considered useful if samples are taken within a few days of the exposure as most forms of Hg in the blood decrease by one-half every three days if exposure has ceased.

As for manganese (Mn), systemic homeostasis of this essential element is tightly maintained under normal dietary consumption by both its rate of intestinal absorption and its efficient removal by the liver. As these processes keep Mn levels in an optimum range for nutritional requirements of the body, blood or urine biomonitoring can be considered unreliable. However, this delicate system for *in vivo* Mn regulation may fail under chronic high doses exposure conditions (Roth, 2006). In workers, group average blood levels appear to be related to Mn body burden, while group average urinary excretion levels are considered to be most indicative of recent exposures. Blood and urine levels may also be useful in detecting groups with above-average current exposure to Mn.

Table 1
Toxicological significance and major advantages and limitations of the biological samples used for human biomonitoring (HBM) of metallic and metalloid elements.

Biological samples used for HBM	Toxicological significance/ relevance	Advantages	Limitations/Precautions	Reference/HBM values
Accepted				
Blood	Recent exposure (except for Methyl-Hg)	Central body compartment in steady state with organs	Complex matrix (proteins, lipid, cells)	Wilhelm et al. (2006)
	Extensive use for Pb		Invasive technique (children)	
	Environmental and occupational exposure		Positive results in the absence of adverse effects (D'Ilio et al., 2013)	
			Anticoagulant interferences	
Urine	Recent exposure (except for Cd)	Non-invasive biological matrix (children)	Complex matrix	McDowell et al. (2004); Morais et al. (2012); Schulz et al. (2009) http://www.cdc.gov/exposurereport/pdf/fourthreport_updatedtables_aug2014.pdf [accessed 19 December 2014]
	Interest of chemical speciation (As, Hg)		Polyethylene containers pre-treated with HNO_3	
	Environmental and Occupational exposure		Normalize for urine creatinine concentration (Barr et al., 2005)	
			Food interferences (e.g. seafood for As, Hg) (Olmedo et al., 2013)	
Not conventional				
Hair	Chronic exposure (related to the growth period)	Non-invasive, easily accessible and stable matrix (children)	External contamination	Druyan et al. (1998)
	Environmental exposure (Hg, As)	Simple collection and transportation	Hair treatment (dyeing, breaching, waving or deodorant)	
		Lack of storage changes	Need of a cleaning-up step (Olmedo et al., 2010)	
		Allows repeated determinations over time	Influence of confounding factors (age, sex, color, diet, hair care, smoking, ethnic factors…) (Esteban and Castaño, 2009)	
		Segmental hair analysis	Useful for Hg (Methyl-Hg) and As (ATSDR -Agency for Toxic Substances and Disease Registry-, 2001; Harkins and Susten, 2003)	
		Higher levels than those found in blood or urine	Complex incorporation-kinetics	
			Absence of data to predict adverse effects in health; correlation between hair and blood for Pb and Cd are divergent (Esteban and Castaño, 2009)	
Saliva	Limited use for environmental exposures	No influence of orthodontic treatment on trace metal levels (Martín-Cameán et al., 2014; Mikulewicz et al., 2011)	Variation in salivary flow-rates	Absence of reliable reference values (Barbosa et al., 2005; Esteban and Castaño, 2009)
			Intra/inter-individual variation in whole saliva composition	
			Potential blood contamination during collection	
			Lack of certified reference materials	
			Influence of confounding factors (nutritional and hormonal status…) (Barbosa et al., 2005)	
			Presence of very low concentrations	
			Precautions before sample collection (Kim et al., 2010; Olmedo et al., 2010)	
Placenta	Prenatal exposure to trace metals and risk assessment	Non-invasive biological sample	Lack of information on potential sources of exposure (chemicals, dietary intake, life style habits…)	Not established (Al Saleh et al., 2011; Esteban-Vasallo et al., 2012)
	Environmental exposure	Placenta levels are higher than those found in maternal and cord blood (Al Saleh et al., 2011; Marques et al., 2007; Needham et al., 2011; Soria et al., 1992)	Precautions before sample analysis (Amaya et al., 2013; Esteban-Vasallo et al., 2012)	
			Low concentrations of metal elements in placenta should never be considered safe for the fetus	
Meconium	Cumulative prenatal exposure to trace metals during 12–40 weeks of gestation (Lisowska-Myjak, 2005)	Easy to obtain and a non-invasive collection	Differences in the presence of metallic elements between meconium and blood (Esteban and Castaño, 2009)	Not established (Lisowska-Myjak, 2005; Ostrea et al., 2006; Turker et al., 2013)
	Environmental exposure	Better analytical sensitivity than urine or cord blood	Need of lyophilization and digestion prior to analysis (Yang et al., 2013)	
		The most reliable indicator of chemical exposure to the fetus during gestation		
Nail	Chronic exposure (integration of growth rate)	Not requiring special storage conditions	Influenced by environmental and nutritional factors: direct contact with amalgams, smoking habits, fish consumption (Esteban and Castaño, 2009; Lemos and de Carvalho, 2010)	Not established
	Environmental exposure (Ndilila et al., 2014)		Toenails are less affected than fingernails by contamination and may provide a longer exposure window (Barbosa et al., 2005)	
			Need of pretreatment before analysis	
Teeth	Chronic exposure – Children (integration of growth period)	Non-invasive biological sample	Not readily available	Not established
	Environmental exposure	Shed teeth allow for assessing exposure during early development (Abdullah et al., 2010)	Can be affected by caries, roots and by tooth group (Tvinnereim et al., 2000)	
Breast milk	Indicator of prenatal exposures to bioaccumulative chemicals		Influence of dietary habits and other factors (Leotsinidis et al., 2005)	Not established
	Environmental exposure (Massart et al., 2008)		Lower levels than those found for blood	

2.2. Urine

Although urine is a fluid currently used for HBM studies (Table 1), its high concentration of inorganic salts makes it a difficult matrix for analytical purposes. Urine usually provides information on recent exposure, so that it is not useful for determining whether chronic exposure has occurred to metallic elements except for those with long half-lives (e.g. Cd). For general population sampling, where the exposure to metallic elements is expected to be low and likely uniform over time, urine biomonitoring can fairly reflect exposure. Thus, major biomonitoring studies conducted in children (e.g., NHANES, GerES studies) have used urine instead of invasive samples, such as blood, as the latter are difficult to obtain and would result in low participation rates potentially leading to selection bias (McDowell et al., 2004).

First morning urine samples should be collected and stored at least at −20 °C until analysis for less than 3 months. Oral instructions focusing on precautions against contamination should be always given before sample collection (Gil and Hernández, 2009).

As sometimes the knowledge of the total content of metallic and metalloid elements in a biological sample does not give a complete value of the environmental situation, it is necessary to carry out a chemical speciation analysis to evaluate properly the toxic potential. Measurement of urinary As levels is generally accepted as the most reliable indicator of recent As exposure because urine is the main route of excretion of most As species. About 75% of total urinary As consists of organics forms (Caldwell et al., 2009) and total urinary As levels have been accepted as a good biomarker of dose (WHO, 2001). Thus, total urinary As is a less useful biomarker of exposure to inorganic As, unless ingestion of foods of marine origin (e.g., especially seaweed and mussels) can be excluded as such foods can result in a rapid increase in total urine As levels. Even small amounts of seafood can invalidate total urinary As as an indicator of exposure to inorganic arsenic, particularly if such intake is common and the exposure to inorganic As is low. In such a scenario, people should be asked to refrain from eating such food at least 5 days before urine sampling. To overcome these limitations, the sum of inorganic species and their metabolites should be routinely measured.

Hg in urine is currently used to test for exposure to metallic Hg vapor and inorganic forms of Hg. Besides industrial emission, Hg levels are most likely due to the large use of dental amalgam fillings, and methylmercury exposure derives also from fish consumption (WHO, 1990). However, urine is not useful for determining whether exposure has occurred to methyl-Hg. Hg levels found in urine, blood and hair may be used together to predict possible health effects that may be caused by the different forms of Hg (ATSDR -Agency for Toxic Substances and Disease Registry-, 1999).

Although measurements of urinary Pb levels have been used to assess Pb exposure, they are not as reliable as analysis in whole blood, which is the most common and accurate method of assessing recent Pb exposure (ATSDR -Agency for Toxic Substances and Disease Registry-, 2007). Urinary Pb concentration increases exponentially with blood Pb and can exhibit relatively high intra-individual variability, even at similar blood Pb levels. However, the use of non-invasive biological samples (such as urine or hair) may provide useful information for biomonitoring studies in certain subgroups of population (e.g., children) where blood testing is more difficult to carry out.

Low levels of essential elements such as Mn are non-toxic but at high levels may produce adverse health effects. Urine (and also blood) levels may be useful in detecting groups with above-average current exposure to Mn. However, measurements in individuals may only be related to exposure dose after exposure has ceased (ATSDR -Agency for Toxic Substances and Disease Registry-, 2012). Because excess Mn is usually removed from the body within a few days, past exposures are difficult to measure with common laboratory tests.

The urinary Cd concentration is mainly influenced by the body burden and is proportional to the concentration in the kidneys. The amount of Cd in urine shows both recent and past exposure (ATSDR -Agency for Toxic Substances and Disease Registry-, 2012). Non-smokers have urinary Cd concentrations of 0.02–0.7 μg/L, and their levels slowly increase with age together with the accumulation of Cd in the kidney (Berglund et al., 2011). While tobacco smoke is one of the largest single sources of Cd exposure in humans, food products comprise most of the human exposure burden to Cd in non-smokers and non-occupationally exposed workers (Morais et al., 2012). The revised German reference level for urine Cd is 0.2 μg/L (Schulz et al., 2009); accordingly, levels above this value are a matter of concern as Cd is a known carcinogen. Besides, values of urine Cd above 1 μg/g creatinine have been associated with renal tubular dysfunction, calcium metabolism disturbances and a higher risk of lung cancer (Banza et al., 2009).

2.3. Hair

Despite blood and urine analysis being traditional approaches for evaluating levels of metallic and metalloid element in the human body, these compounds are steadily incorporated into hair over time. In fact, as metal or metalloid cations bind to the sulfur atoms of the keratin present in hair matrix (Bencko, 1995), hair can be used as a screening tool for biomonitoring chronic exposure to environmental toxic compounds. Hair concentrations of metallic and metalloid elements are up to 10-fold higher than those found in traditional samples of blood or urine (Bader et al., 1999; Gammelgaard and Velen, 1990). The metallic element composition of hair has two major sources: secretion of endogenous compounds from the bloodstream and uptake from the external surface of the hair because of external contamination. As hair is hygroscopic, a number of liquid materials (shampoos, dyes and even sweat) containing metallic elements can enter the hair. This process appears to be pH sensitive and similar to ion exchange, but shows wide interindividual differences (Buckley and Dreosti, 1984).

Human hair has a number of advantages over other biological samples (Table 1). Hair grows approximately 10 mm per month and allows for long-term monitoring of past and recent exposure and usually reflects average concentrations for the previous months' exposures (Bermejo-Barrera et al., 1998). Although segmental hair analysis (e.g. determination of the analyte concentration along the length of the hair) might provide valuable information on the time of exposure, it is not currently carried out for biomonitoring purposes in the general population (e.g. NHANES and GerES studies).

ATSDR has explored human hair analysis as a potential additional tool to assess exposure and the US-EPA has regarded hair as one of the most important biological materials for worldwide environmental monitoring of elements. Hair has also been used by the International Atomic Energy Agency (IAEA) to monitor trends in element levels (Druyan et al., 1998; Morton et al., 2002). Although analytical methods can detect trace or low amounts of metallic and metalloid elements in the hair and could indicate exposure (ATSDR -Agency for Toxic Substances and Disease Registry-, 2001; Harkins and Susten, 2003), there is controversy on whether the measurement of a substance in the hair accurately reflects external exposure or internal body dose. Except for Hg, methyl mercury, (and perhaps As), data are insufficient to reliably indicate the source of exposure and the internal dose as well as to predict the resultant health effects from the measurement of a particular substance in the hair (ATSDR -Agency for Toxic Substances and Disease Registry-, 2001; Harkins and Susten, 2003). The presence of roadside dust in hair and evaporation of sweat on hair could lead to an even greater incorporation of metallic and metalloid elements in hair via this

exogenous route. In addition to the potential for external contamination, other limitations for hair testing are listed in Table 1. To avoid this drawback, hair samples (50 mg) must be subjected to the washing procedure recommended by IAEA to provide an accurate assessment of the endogenous content of metallic and metalloid elements (Bermejo-Barrera et al., 2000; Olmedo et al., 2010). In particular, hair must be initially washed by ultrasonic cleaning in a non-ionic detergent solution (e.g. Triton X-100). Thereafter, the detergent is removed by ultrapure water and then hair must be washed by ultrasonic cleaning in an ethanol solution. Microwave digestion procedure using a HNO_3–HCl–H_2O_2 mixture has been recommended as the sample preparation method. Preliminary experiments must be performed to check that scissors do not release metal elements; nevertheless, ceramic scissors can be used instead to avoid metallic contamination. As hair readily adheres to the wall of containers by electrostatic attraction, care must be taken in transfer operations (Olmedo et al., 2010).

Another disadvantage of hair testing is the lack of sufficient information to define a reference range for the general population, because concentrations of metallic and metalloid elements in hair vary significantly (Esteban and Castaño, 2009) (Table 1). Thus, more studies are needed to broaden the database for these compounds in hair with the aim of setting reference values that allow hair testing to become a reliable exposure biomarker. Certain authors have suggested analyzing axillary hair instead of the more common scalp hair because the armpits are much less prone to external contamination by metallic and metalloid elements present in dust (Bader et al., 1999; Gil et al., 2011).

The correlation between levels of these elements in hair and other biological materials commonly used for environmental exposure assessment, such as blood or urine, has been previously addressed for biological monitoring purposes. Circulating levels of metallic and metalloid ions can be attached to the keratin molecules during the short period of hair formation and theoretically should correlate with their blood concentration (and perhaps, body stores) during hair formation (Gil et al., 2011). However, while hair content for most metallic and metalloid elements is usually related to past exposures, urinary levels reflect very recent exposure. The lack of significant correlation between the content of these elements in blood and other samples (e.g. hair) might be due to their different and rather complex incorporation-kinetics in these other compartments.

There is general consensus that many scientific issues need to be resolved before hair analysis can become a useful tool in understanding environmental exposures. Besides, the range of metal and metalloid contamination levels typically found in human hair is unknown for the scientific community. Without reliable data on baseline or background hair contamination levels in the general population, health agencies cannot determine whether results from a given site are unusually high or low. In addition to the pre-analytical issues and the absence of reliable reference ranges, the quality of analytical techniques used for determining metallic and metalloid elements in hair has been questioned.

Only As and Hg are validated to be measured in hair (ATSDR -Agency for Toxic Substances and Disease Registry-, 2001; Harkins and Susten, 2003). Hair Pb is a relatively poor predictor of blood Pb, particularly at low concentrations. The concentration of As in the root of the hair is in equilibrium with the concentration in the blood. Studies of exposed workers have not found a quantitative relationship between hair Cd levels and body burden. Because of the potential for sample contamination, hair levels are not reliable either as predictors of toxicity or as indicators of occupational exposure.

More research is needed to establish standardized references ranges, gain a better understanding of hair as a better measure or predictor of disease than other traditional biological samples.

2.4. Saliva

The presence of metallic and metalloid elements in saliva has been reported by several authors (Agaoglu et al., 2001; Burguera et al., 1998; Gil et al., 2011; Menegário et al., 2001; Wang et al., 2008). Although saliva could be pursued as an alternative biological sample to blood and urine for metallic and metalloid elements testing, it has not yet been accepted as a useful tool for biological monitoring (e.g. occupational purposes). A number of factors have limited the use of saliva as a suitable sample for occupational and environmental exposures. Major limitations of saliva as a matrix for testing metallic and metalloid elements are listed in Table 1. Besides, saliva shows large variations in its ion content throughout the day, coupled with changes in salivary flow rates before, during, and after meals. Variations also arise depending on the manner in which saliva collection is stimulated (or not) and on the nutritional and hormonal status of the individual (Barbosa et al., 2005; Esteban and Castaño, 2009).

One of the reasons for the scarcity of studies carried out in saliva could be related to the fact that this matrix is physiologically and biochemically a heterogeneous fluid whose composition reflects that of typical extracellular fluids (Wang et al., 2008). Using the whole (mixed) saliva overcomes some of the problems caused by variations in its composition depending on the gland where it is produced. Whole saliva also has the advantage of reflecting biological events occurring in the mouth for much of the day (Duggal et al., 1991). The physical properties, amount, and composition of saliva are influenced by factors such as diet, time of day and physic condition and all these factors may possibly explain the variations in saliva composition (Fors and Persson, 2006; Kocadereli et al., 2000). These fluctuations, along with the ionic composition of saliva, may also affect the behavior of metallic and metalloid elements in the mouth.

Moreover, an increase in the salivary concentration of certain metal compounds (e.g. nickel and chromium) has been reported following the insertion of fixed orthodontic appliances, particularly following bonding (Fors and Persson, 2006). These appliances might increase the amount of metallic and metalloid elements in saliva. The presence of haemoglobin may also affect the values of certain toxic metals (Gjerdet et al., 1991).

When using saliva as biological matrix for testing metallic and metalloid elements, samples should be collected before breakfast to minimize variability in salivary composition and immediately after rinsing the mouth with sterilized distilled water. Subjects are requested to refrain from oral activities, such as chewing, drinking, tooth brushing, and cigarette smoking for at least 1 h before sample collection (Kim et al., 2010). Unstimulated saliva can be collected in polypropylene tubes, and the first milliliter should be discarded. After centrifugation to precipitate sediment cellular debris, samples should be stored at −20 °C until processing (Olmedo et al., 2010).

2.5. Placenta

Fetuses and neonates are especially vulnerable to toxic chemicals because of the immaturity of their detoxification systems and high rate of cell proliferation and tissue growth. As the placental passage represents the main access of chemical agents to the fetus, placenta samples can be helpful to investigate intrauterine exposure to metallic and metalloid elements. Placenta is easy to obtain and may furnish information on the exposure of both mother and fetus (Esteban-Vasallo et al., 2012). Because some metallic and metalloid elements may reach and cross the placental barrier and interfere with placental transport systems, prenatal exposure to these toxic compounds is a matter of special concern (ATSDR -Agency for Toxic Substances and Disease Registry-, 2007; Osman et al., 2000; Wier et al., 1990; Zhang et al., 2004).

As the placenta accumulates metallic and metalloid elements during pregnancy, it can be used to assess chronic exposure to these compounds, thus avoiding repeated maternal blood sampling and other invasive biomonitoring (Esteban and Castaño, 2009); the role played by placental xenobiotic metabolizing enzymes makes this sample a very sensitive matrix to examine the fate and effects of chemicals on the fetus.

Major limitations and precautions of placenta are shown in Table 1. Prior to analysis, the placenta needs an initial washing to remove blood or clots and then is homogenized and digested. Some data indicate that pre-treatment, elimination of fluids, and freezing may modify levels of metallic and metalloid elements (Esteban-Vasallo et al., 2012). For these compounds, some studies have found a positive correlation between placenta and venous or arterial cord blood, umbilical tissue or maternal and neonate hair (e.g. Hg) (Needham et al., 2011). On the other hand, smoking during pregnancy significantly contributes to higher Cd concentrations in placenta (Amaya et al., 2013; Stasenko et al., 2010).

Further research will permit the investigation of the factors contributing to intrauterine exposure to metallic and metalloid elements, the impact on infant development of the placental transfer of toxic compounds and the establishment of validated reference levels.

2.6. Meconium

Due to placental transfer, measurement of contaminants in biological samples from the newborn may reflect *in utero* exposures. The prenatal period is a critical time when the central nervous system is developing, and the fetus is particularly sensitive to chemical exposure during this stage. Meconium is the first, viscous, odorless and black feces discharged 2–3 days after birth. It is formed by the fetus as early as the 12th week of gestation and accumulates throughout pregnancy (Ostrea et al., 2006). As meconium is not excreted before birth, it can be used to examine cumulative prenatal exposures during the second and third trimesters of pregnancy. A narrower time window of exposure cannot be used. Major advantages and limitations of meconium as a biological matrix for HBM are shown in Table 1. This biological medium has demonstrated its usefulness for the analysis of nicotine, drugs, alcohol and environmental contaminants. Meconium, as well as placenta and cord blood, are pertinent media for assessing the actual body burden of metallic and metalloid elements in neonates. In addition, serial collection of meconium for a newborn may reflect the degree and time of maternal exposure at various stages of gestation (Ostrea et al., 1994). In this regard, Ortega-García et al. (2006) divided meconium into three parts: 0–10 hours, 10–24 hours, and 24–48 hours after birth, where concentrations of some environmental contaminants increased from the first meconium stool to the third, which might allow the timing of the highest exposure of the fetus to be estimated. Furthermore, the analytical sensitivity for many compounds is better than when using urine or cord blood samples, so that meconium appears to be a helpful tool in evaluating fetal exposure to environmental contaminants that might affect children's development (Tuomisto, 2006).

Although metallic and metalloid elements exposure assessment during the fetal period has been carried out mainly using cord blood, meconium is an alternative sample for identifying *in utero* exposure of infants to a number of illicit and legal drugs, pesticides and metallic and metalloid elements (Jiang et al., 2010; Maynard et al., 1991; Ostrea et al., 2002; Turker et al., 2006). As the behavior of chemical concentration in meconium differ from other samples, more research is warranted to compare with the concentration of metallic and metalloid elements in other biological samples (cord blood, placenta) for a proper assessment of the usefulness of meconium in the evaluation of prenatal exposure to those compounds (Yang et al., 2013).

Meconium samples should not contain toxic metal and metalloid elements under normal conditions. The fetus can be exposed to these and other chemicals, most of which are deposited and accumulated in the meconium. These chemicals can be accumulated in meconium through bile secretion and/or fetal swallowing of amniotic fluid, which contains these chemicals (Ostrea et al., 2006). Meconium appears to be the most reliable matrix for assessing fetal Hg exposure, even though Hg levels in maternal blood and cord blood are low (Lanphear and Bearer, 2005; Ramirez et al., 2000). Some studies have shown an association between preterm mortality and concentrations to metallic and metalloid elements (e.g. Pb) in newborn meconium (Turker et al., 2013).

2.7. Nail

Usually, 0.5 g of nail in weight is required for laboratory analysis. Depending on the length of the nail sample, a window of exposure between 1 and 6 months can be assessed. Chemicals are deposited in the nails through the blood stream following inhalation or ingestion; however, they may undergo metabolism and can generally be identified in the nail tissues 1–2 months after being absorbed based on growth rate.

Advantages and limitations of nail samples are listed in Table 1. As toenails have a slower growth rate than fingernails (up to 50% slower, especially in winter), they provide a longer integration of exposure to environmental chemicals, in particular to Pb exposure (Barbosa et al., 2005).

When external contamination can be removed, toenail metallic and metalloid elements concentrations are generally considered reflective of internal body stores and they can also be useful indicators of environmental contamination (Garland et al., 1993; Lauwerys and Hoet, 2001; Slotnick and Nriagu, 2006). In fact, elevated toenail concentrations of metallic and metalloid elements have been found in mining areas with significant environmental exposure to these compounds (Ndilila et al., 2014). Nail samples are usually pre-cleaned by ultrasonic agitation in a Teflon beaker containing a solvent (e.g. acetone), dried at room temperature and then, like hair, processed using acid digestion in a microwave oven in order to convert into liquid prior to the measurement of metallic and metalloid elements (Lemos and de Carvalho, 2010).

Gulson (1996) found that the Pb concentration in nail has a very high intra-individual variation so that this matrix seems not to be suitable for biomonitoring purposes. However, due to their low growth rates, fingernail and toenail Hg levels are reliable indicators and can serve as a reflection of Hg exposure over the previous year (Daniel et al., 2004).

2.8. Teeth

Teeth have been used as indicators of exposure to metallic and metalloid elements for several decades. However, teeth are not appropriate for biomonitoring because they are not readily available. When available, they can be used as a biological matrix for assessing the burden of metallic and metalloid elements. Shed teeth are obtained non-invasively and provide a window into archival levels of exposure to these compounds during early periods of development (Abdullah et al., 2010). Additional advantages and limitations of teeth as a biological matrix for HBM are shown in Table 1. Pb levels in teeth have been used as a measure of exposure to this metal, especially in growing children (Gil et al., 1994).

2.9. Breast milk

While breastfeeding has been recognized and promoted by public health officials as the most beneficial source of nourishment during infancy, it can be a potential source of exposure to toxic chemicals

to which the mother has previously been exposed. Chemicals accumulated in the mother's tissues may be transferred to infant during breastfeeding (Massart et al., 2008). Although infants are likely to be exposed to higher levels of metallic elements before birth than during breastfeeding, breast milk may be an additional pathway of exposure (Table 1). In case of exposure during pregnancy to bioaccumulative compounds, such as some metallic elements and hydrophobic organic chemicals, breast milk might provide information on prenatal exposure to these compounds (Needham and Wang, 2002; Solomon and Weiss, 2002).

Detectable levels of toxic metals such as Pb, Hg and Cd have been documented in breast milk population studies of women with no current environmental or occupational exposures. A number of breast milk monitoring studies have reported a wide range of variation for mean values of toxic metals in human milk (Al-Saleh et al., 2003; Dorea, 2004; Leotsinidis et al., 2005).

Unlike other persistent contaminants, metallic and metalloid elements (e.g. Pb, Hg and Cd) appear in human milk at lower concentrations than lipid-soluble chemicals and their levels represent about 20% of the those found in blood from the same person (Golding, 1997). This is attributed to their low lipid-solubility and high binding to erythrocytes.

Levels of metallic and metalloid elements in breast milk are significantly associated with heavy road traffic and industrial activity. Lactation requires a substantial redistribution of maternal calcium that is mobilized from bone stores. As Pb accumulates in bone from past exposures, during lactation it can be released into blood and excreted into breast milk (Ettinger et al., 2004).

3. Validation of analytical procedures for biomonitoring of metallic and metalloid elements in biological samples

Different methodological approaches (sampling, sample preparation, analytical methods, etc.) have been used for the determination of metallic and metalloid elements in biological matrices. In consequence, results from different studies are difficult to compare and meaningful conclusions cannot be reliably drawn (Dongarrà et al., 2011). Validation of analytical procedures has become a routine requirement for reporting data pertaining to human biomonitoring. Analytical issues certainly need to be taken into account when evaluating data reported in older studies.

The analysis of metallic and metalloid elements in biological matrices, other than blood and urine, has not yet been accepted as a useful tool for biological monitoring, so that the validation of analytical procedures in these non-conventional samples is absolutely relevant. The most significant problems encountered in the laboratory methodology used for non-conventional samples analysis include variation in sample preparation, variation in laboratory quality control programs and interlaboratory variability in reference ranges, results and interpretation of data (Harkins and Susten, 2003).

Methodologies for the determination of the metallic and metalloid elements have been generally reported for blood and urine, but are scarce for other matrices such as placenta, saliva, meconium, etc. Validation of the analytical methods has become a basic prerequisite for official laboratories involved in biomonitoring of metallic and metalloid elements (Gil et al., 2006; Olmedo et al., 2010). According to the recommendations of IUPAC (International Union of Pure and Applied Chemistry) and other agencies (UNI CEI EN ISO/IEC 17025, 2005; Commission Decision, 2002, 657/EC, 2002), the validation protocol must include the following parameters: limit of detection (LOD) and quantification (LOQ), linear range, precision (minimal, intermediate and reproducibility), accuracy, recovery and characteristic mass. Furthermore, the analytical method must be always controlled by using external certified reference materials (CRM). As certified reference materials are not available for metallic and metalloid elements for all biological samples (e.g. saliva or placenta) the accuracy is sometimes replaced by recovery studies using samples spiked with a standard solution of these elements. In these cases, recoveries must demonstrate a good concordance with respect to theoretical values.

In many cases, prior dilution of samples is a critical step and the best results are obtained by using appropriate dilution factors. These factors must be adequately elucidated by studying the dilution effect on the analyte concentration. The mere dilution of some specific biological samples (e.g. saliva or urine) could be enough to minimize the interference and effects of the biological matrix such as proteins, salts and other compounds (Kocadereli et al., 2000; Olmedo et al., 2010). Furthermore, the methods validated in a given laboratory should be checked and revalidated whenever they are adopted by another laboratory.

Particular attention must be paid to the number of samples with levels of metallic and metalloid elements below the LOD. As their concentration in hair and urine do not fit a normal distribution, range, median, percentiles (25, 75, 95th) and geometric means should always be provided. According to the CDC (2009), concentrations less than the LOD can be assigned a value equal to the LOD divided by the square root of 2 for calculations.

A high percentage of samples below the limit of quantification makes the comparison with other studies particularly difficult. As a general rule, if more than 60% of samples from a study population are below the LOD, percentile 25, median and geometric mean statistics cannot be calculated. This drawback can be partially overcome by using Tobit regression models, which is the recommended approach (Molina-Villalva et al., 2015). These models require specifying the limit below which the actual value of the dependent variable (levels of metallic and metalloid elements) is unknown, so they allow for an estimate of the effect, despite having a large number of samples below the LOD. Thus, Tobit models are useful for modeling censored variables and suitable for measurements with a detection limit below which the actual value of the variable is unknown. These models take into account the uncertainty in the estimates, which will be greater as the number of censored values increases. However, a large number of values below the LOD affect the standard error of the estimate and consequently the confidence interval. The results of the multivariate analysis are valid because the data have been analyzed using techniques that make an adequate treatment of these censored data, and uncertainty has been considered for the estimates.

Traditionally, urinary biomonitoring data are adjusted to a constant creatinine concentration to correct for variable dilutions among spot samples. While this approach has been used in population groups without much diversity, the inclusion of multiple demographic groups in biomonitoring studies for exposure assessment has increased the variability in the urinary creatinine levels. To overcome this variability, multiple regression analyses are recommended to include the analyte concentration (unadjusted for creatinine) along with urinary creatinine added as a separate independent variable (Barr et al., 2005). Because the World Health Organization guidelines consider that urine samples with creatinine values less than 0.3 g/l or greater than 3 g/l are unrepresentative, they should be excluded from statistical analysis to minimize both over- and underestimation in calculating the normalized concentrations.

4. Influence of potentially confounding factors on metallic and metalloid elements concentration

A specific questionnaire must be always administered to the study participants to obtain information on sex, age, body mass index (BMI) and life-style habits (smoking, alcohol consumption and food intake frequency). All these factors may contribute to variation in metallic and metalloid elements concentration (Gil et al., 2011; Leung and

Huang, 1997). Fish, nutrient supplement and medicinal herbs intake are particularly relevant in relation with exposure to those compounds (Hsi et al., 2014).

Hair levels of metallic and metalloid elements tend to vary from one geographical region to another depending on the natural background conditions, including composition of soil, element concentration in water and food and eating habits. Therefore, the analysis of biological samples should always include a dietary questionnaire that provides information on the potential source of body burden of metallic and metalloid elements (Bencko, 1995; Fors and Persson, 2006). For instance, cereals and vegetables represent the major source of Cd exposure for the general population. Also, type of water consumption may have an impact on As levels and certain fish species are the major source of Hg.

Importantly, metallic and metalloid elements speciation is relevant for the interpretation of analytical results for some of these compounds. In this regard, Hg is an element of special concern because its inorganic form is biologically transformed in aquatic environments into methylmercury (MeHg), a lipophilic organic compound that bioaccumulates and biomagnifies as it moves up the aquatic food chain, particularly in predatory fish species (e.g. tuna) (Gewurtz et al., 2011; Olmedo et al., 2013). Several studies have shown that higher Hg levels in hair are likely related to fish consumption as contaminated fish represent the primary route of exposure to methylmercury (Benefice et al., 2008; Peplow and Augustine, 2012). Even though total Hg is currently measured in most epidemiologic studies in the general population, 80% of total hair Hg consists of methylmercury and both of them are linearly related (McDowell et al., 2004). Measurement of Hg in whole blood or scalp hair is used to monitor exposure to methyl-Hg; by contrast, urine is not useful for determining whether exposure has occurred to methyl-Hg. Hence, hair can be a useful matrix for determining Hg in the general population but is less relevant for individuals occupationally exposed to Hg. In this case, urine Hg is currently used to test for exposure to metallic Hg vapor and to inorganic forms of Hg. Nonetheless, levels found in blood, urine and hair may be used together to predict possible health effects that may be caused by the different forms of Hg (ATSDR -Agency for Toxic Substances and Disease Registry-, 1999).

With regard to As speciation, it is well-known that most As found in fish and shellfish is organic As, the less toxic form. According to the European Food Safety Authority (EFSA, 2009), only 2% and 3.5% of the As contained in fish and shellfish products, respectively, could be considered as toxic inorganic As. In other circumstances, As concentrations in drinking water (particularly from well or spring) could influence As urine levels (Molina-Villalva et al., 2015).

Hair content of chemicals depends on age, sex (Chojnacka et al., 2006a; Senofonte et al., 2000), living habits (Chojnacka et al., 2006b; Miekeley et al., 1998), and environmental exposures (associated with urbanization and industrialization) (Ashraf and Jaffar, 1997; Gil et al., 2011). Girls usually exhibit higher concentrations of metallic and metalloid elements in hair than boys (Batista et al., 1996; Fujimura et al., 2012 and Menezes-Filho et al., 2009; Molina-Villalva et al., 2015), a finding that can be accounted for by the different growth rate of hair among individuals since a larger metallic and metalloid elements accumulation is expected with a slower rate of hair growth (Sanna et al., 2008). Nevertheless, there are some controversial results as greater Hg and Pb levels have been reported for males as compared to females (Olivero-Verbel et al., 2008; Sanna et al., 2003).

Questionnaires should also include data on health status (absence of serious chronic diseases such as diabetes, hypertension or thyroid disease), medication and history of metal allergy, orthodontic appliances as well as occupational data such as lifetime working experience (years), hygiene conditions and use of personal protective equipment (e.g. face masks, respirators, etc.). It is also important to assess the place of residence (e.g. urban, rural, etc.) (Molina-Villalva et al., 2015).

In summary, there is a need for information on exposure, lifestyle and socioeconomic factors, stratified by gender and age, for the purpose of conducting balanced risk assessment and management that considers such differences (Berglund et al., 2011).

5. The importance of setting reference values for biomonitoring of metallic and metalloid elements in non-conventional biological samples

Reference values concern healthy subjects and use reference groups and are also called normal ranges or normal values. Specific ranges are usually made for sex, age group, race, etc. (Mikulewicz et al., 2013; Poulsen et al., 1997). Reference values for environmental pollutants in human biological materials (e.g., blood, urine) are derived following the methods and criteria currently used to establish reference values for clinical chemistry parameters. Generally, reference values are based on analyses of blood or urine collected from a representative sample of the general population. Hence, they are statistically derived values and do not represent health-based guidance values. If a measured pollutant concentration in blood or urine exceeds the reference value, it does not necessarily mean that there is an increased health risk (Schulz et al., 2007).

The utility of exposure and biological effect measures has become increasingly important to establish safety environmental concentrations for metallic and metalloid elements in order to prevent early harmful effects on humans. One of the most important issues in using alternative samples for HBM studies is the definition and development of "reference ranges" that describe exposure of the general population to metallic and metalloid elements. In contrast to reference values, toxicologically-based values have been established by International Agencies such as NIOSH, OSHA, EPA, ATSDR, etc. for occupational settings and they are used as recommended reference values (Biological Exposure Indices, BEIs) (Ewers et al., 1999; Gil, 2012; Mikulewicz et al., 2013).

The absence of worldwide accepted reference ranges for levels of metallic and metalloid elements in non-conventional samples lies on the lack of a reliable and useful method for assessment exposure to these compounds without any potential interference. Reference range (standard range) is defined as the prediction interval between which 95% of values of a reference group are found. The elaboration of reference ranges usually includes different percentiles, most commonly 5th–95th percentiles.

A systematic review of reference values of elements in human hair has indicated that the lack of reference values is the absence of clear guidance on the methodology for the population selection, sampling, preparation and digestion, as well as analytical procedures. Hence, health-based guidelines for their interpretation have not yet been completely established (Hernández et al., 2014). For hair data, the NHANES 1999–2000 provide reference values for metallic and metalloid elements in U.S. children aged 1–5 years (McDowell et al., 2004). For example, normal concentrations of As in hair of unexposed humans usually ranges from 0.02 to 1 μg/g (Hindmarsh et al., 1999) and 1 μg/g has been set by the WHO (Liu et al., 2010) as the critical value.

6. Future directions

Further research on this issue is needed, especially to ascertain whether or not alternative matrices offer definitive advantages over the traditional biological fluids, blood and urine.

Efforts are needed to broaden the database for metallic and metalloid elements in different matrices that allows for better establishing reference values of non-exposed populations and to

allow them to become accepted reliable exposure biomarkers. The influence of nutritional status should be also considered.

Based on the potential accumulation of many metallic and metalloid elements in certain human tissues, including hair or saliva, the use of these non-conventional biological matrices can be useful for assessing individual or group exposure to those elements as additional or alternative matrices to blood or urine for population biomonitoring programs.

For hair to be considered as a reliable index of chronic exposure, a quantitative relationship between hair levels and body burden must always be found, which is not the case in the available literature. Because of the potential for sample contamination, hair levels are not reliable either as predictors of toxicity or as indicators of exposure. Hair testing can be used as a useful screening tool for biomonitoring chronic exposure to environmental toxic elements and blood testing could be triggered in cases of excessive exposure, thus allowing for an improved assessment of the long term risk for the general population (Bencko, 1995).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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